EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure
for
Neutralization Confirmation Procedure for Products Evaluated with the AOAC Sporicidal Activity Test (*Bacillus* Species)

SOP Number: MB-12-00

Date Revised: 02-06-02

Prepared By: _____ Date: ___/___/___

Print Name:_____

Reviewed By: _____ Date: ___/___/___

Print Name:_____
Technical Staff

_____ Date: ___/___/___

Print Name:_____
QA Officer

_____ Date: ___/___/___

Print Name:_____
Laboratory Director

Date Issued: ___/___/___

Withdrawn By: _____ Date: ___/___/___

Controlled Copy No.: _____

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1.0 SCOPE AND APPLICATION:

1.1 The neutralization of the active ingredients found in antimicrobial products is one of the most important steps in efficacy testing. A neutralizing agent is used in each test to inactivate the product's active ingredients, a process essential to achieving the desired contact time. Bacteriostatic activity may bias the outcome of an efficacy evaluation.

1.2 This SOP describes methodology which will be used to determine the effectiveness of neutralizers specified for sporicidal activity testing. The OPP Microbiology laboratory utilizes the FDA Protocol for Testing Sporicidal Activity (June, 1993), a protocol based on the official AOAC Method 966.04, 15th edition (see ref. 15.1). The FDA protocol (Section IV, B) contains instructions for selecting a suitable neutralizer. However, the FDA method is a qualitative assay that uses seeded carriers (carriers used in sporicidal testing), and does not provide for a range of spore challenge. SOP MB-12 is a carrier-based method (sterile carriers) which simulates the test conditions, but is designed to quantitatively assess the effectiveness of neutralizers across a broad range of spore concentrations.

1.3 In most cases, *Bacillus subtilis* (ATCC #19659) will be the test microbe selected for sporicidal testing, and will be used in the neutralization testing; however, if requested, other *Bacillus* species may also be used.

1.4 This method can also be used to determine the effectiveness of an alternative neutralizer, one not specified in the test parameters.

1.5 It is preferable to perform the neutralization assay concurrently with product testing; however, an independent, stand-alone assay may also be performed.

2.0 DEFINITIONS:

2.1 AOAC = AOAC INTERNATIONAL

2.2 CFU = Colony Forming Unit

2.3 PBDW = Phosphate Buffered Dilution Water

2.4 TSA = Tryptic Soy Agar

2.5 FTM = Fluid Thioglycollate Medium

2.6 DI = Deionized Water

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organism (*Bacillus subtilis*) are required to be performed in accordance with biosafety practices stipulated in the SOP MB-01 (see ref. 15.2). Biosafety level 2 practices will be followed for tests involving *Bacillus subtilis*; however, the appropriate biosafety practices must be addressed for individual microbes.

3.2 Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Latex gloves and other personal protective clothing or devices must be worn during the handling of these items for purposes of activation or dilution, or efficacy testing. A chemical fume hood or other containment equipment is employed when performing tasks with concentrated products.

4.0 CAUTIONS:

4.1 To ensure the stability of the test disinfectant, prepare the disinfectant dilutions within three hours of the disinfectant treatment step unless test parameters specify otherwise.

4.2 Strict adherence to the protocol is necessary for validity of test results.

4.3 Use aseptic procedures for all test procedures involving manipulations of the test organisms and associated test components.

5.0 INTERFERENCES:

5.1 For each neutralizer and medium tested per study, one batch (preparation) should be used for all treatment and control groups. Differences in performance (quality) between batches of media may lead to misleading neutralization results.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 For *B. subtillis* (ATCC #19659), a commercial preparation of spores will be purchased to provide inoculum. One source is Presque Isle Cultures, 3804 West Lake Rd., P.O. Box 8191, Erie PA 16505.

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Calibration of equipment used in testing will follow the procedures and schedules outlined in the Laboratory's Standard Operating Procedures for Equipment. These include (see ref. 15.4):

SOP EQ-01	Calibration and Maintenance of pH Meters
SOP EQ 02	Calibration of Thermometers
SOP EQ-03	Calibration and Maintenance of Weigh Balances
SOP EQ-05	Calibration and Maintenance of Timers
SOP EQ-08	Calibration and Maintenance of Automatic Media Dispenser

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Disinfectants are stored according to manufacturers' recommendations or at room temperature if the product label or testing parameters do not identify a storage temperature. Those disinfectants requiring activation or dilution prior to use will only be activated or diluted within three hours of testing unless test parameters specify otherwise.

10.0 PROCEDURE AND ANALYSIS:

10.1 General Description of the Assay. The test conditions specified for product testing (e.g., H_2O hardness, Use Dilution, pH, Organic Soil, Neutralizer, Contact Time, Temperature) must also be followed for the neutralization confirmation assay.

This assay is designed to simulate the conditions of the efficacy test (see FDA Protocol for Testing Sporicidal Activity, June, 1993); however, sterile carriers are used instead of seeded carriers. Diluted inoculum (e.g., spores of *B. subtilis*) is added directly to the various sets of subculture media tubes (see Table 1). The inoculum is quantified by plating on a suitable agar such as TSA. This provides for a quantitative approach to assessing the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer itself or neutralizer x disinfectant interactions.

10.2 Preparation of Inoculum. The inoculum is serially diluted and applied directly to tubes of subculture media.

10.2.1 A concentrated spore preparation may be obtained from a commercial source such as from Presque Isle Cultures, 3804 West Lake Rd., P.O. Box 8191, Erie PA 16505. Based on personal communication, concentrated spore preparations from Presque Isle Cultures are prepared using the following procedure:

- Grow spore forming bacteria on Nutrient Sporulation Agar obtained from a commercial source. Incubate at 37±2°C for a minimum of 5 days.
- Wash the colonies off the surface of the agar with cold sterile deionized water.
- Transfer the colony solution to a 100 ml sterile bottle.
- Wash the spores with 100 ml of cold sterile deionized water. Centrifuge the spores after each wash at approximately 15,000 rpm for 30 minutes. Repeat this step 5 times.
- Transfer the spore using a sterile pipette to a sterile bottle containing 100 ml of an aqueous solution of ethanol (40%).
- Determine the spore count by serial dilution and plating on TSA.
- Store at 5±2°C for up to 1 year.

10.2.2 Initiate serial ten-fold dilutions of the inoculum by pipetting 1 mL of the spore suspension into 9 mL of PBDW or sterile deionized water. Five dilutions, (1×10^{-5}, 1×10^{-6}, 1×10^{-7},

1 x 10^{-8} and 1 x 10^{-9}) will be used to inoculate the neutralizer and subculture media tubes described below. The dilution series is based on an estimate of 10^8 spores per mL for the undiluted suspension. The target number of cells to be delivered is 5-100 CFUs/mL.

10.2.3 To estimate CFUs/mL, plate (pour plate method) each of the five dilutions in duplicate on TSA agar. Briefly vortex each dilution tube prior to plating. See 10.2.6 for pour plate method.

10.2.4 Alternatively, inoculum may be obtained by shearing spores off of commercially available pre-inoculated carriers (inoculated per the AOAC method). One source is Presque Isle Cultures, 3804 West Lake Rd., P.O. Box 8191, Erie PA 16505. Culturing and carrier inoculation steps are described in steps C-3 and C-4 of the FDA protocol. <u>The approx. carrier spore load should be obtained through plating prior to using carriers as a source of spores.</u> To obtain spores from inoculated carriers, perform the following:

- Place an inoculated carrier into a 20 x 150 mm tube containing sterile DI water. Place tube into an appropriately sized beaker and fill the beaker with tap water to the level of sterile DI water in the tube.
- Place beaker containing tube(s) into sonicator; water level in the tank must be at the fill line. Hold the beaker in the sonicator so that it is not touching the bottom, and that the water level inside the beaker is the same as the sonicator tank.
- Sonicate carrier for 5 minutes.
- Vortex the tube for 2 minutes.
- Prepare serial dilutions by transferring 1 mL of the spore suspension (tube with carrier) to a tube containing 9 mL DI water. Dilute the spore suspension out to 10^{-7} and plate (pour plate) the 10^{-3} through 10^{-7} dilutions. See 10.2.6 for pour plate method.
- The five dilutions, (1 x 10^{-3} , 1 x 10^{-4}, 1 x 10^{-5}, 1 x 10^{-6} and 1 x 10^{-7}) will be used to inoculate the neutralizer

and subculture media tubes described below. The dilution series is based on an estimate of 10^6 spores per mL for the undiluted suspension. The target number of cells to be delivered is 5-100 CFUs/mL.

10.2.5 If the product test conditions include the addition of an organic soil load to the inoculum, then the neutralization assay will be performed with the organic soil load added to the inoculum. Otherwise, the inoculum should be prepared without the addition of an organic soil load.

10.2.6 Record the dilution and plating information on the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Serial Dilution/Plating Tracking Form and the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Inoculum Enumeration Form (see 16.0).

10.2.7 Pour plate method: the TSA agar is prepared and tempered (approx. 1 hr) to 45-55°C in a waterbath prior to use. Tempered TSA agar is added to the plate after the addition of the appropriate dilution, and swirled to spread the inoculum.

10.2.8 Incubate plates at 37±1°C for 24-48 hours. Count colonies with aid of a plate counter. Colonies of *B. subtilis* are opaque, rough, dull, round, irregular margins, and low convex. Colonial variation may be observed and is typical for this strain. Plates that have colony counts over 300 can be estimated or labeled TNTC. Record the counts on the Inoculum Enumeration Form for Neutralization Assay (see 16.0).

10.3 Product Sample Preparation.

10.3.1 Follow guidelines for product sample preparation provided by the sponsor for sporicidal activity testing.

10.4 Performing the Assay. The following instructions apply to the analysis of one neutralizer with one carrier type (porcelain penicylinders or silk suture loops). If desired, both carrier types can be evaluated.

10.4.1 Each assay will require five sterile carriers. Use the carrier type required for the specific sporicidal test. Record the test information on the Information Sheet for the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) (see 16.0).

10.4.2 The product must be applied according to specific instructions provided in the test parameters. Expose each carrier to the disinfectant according to the contact time specified in the test parameters for efficacy testing.

10.4.3 Within 5 seconds, a set of 5 carriers is placed into one medication tube containing the disinfectant at time zero or 30 seconds. Transfer carriers according to the method specified in the FDA SOP. Record the carrier transfer information on the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test): Time Recording Sheet for Carrier Transfers (see 16.0).

10.4.4 Allow the carriers to remain in the product per the specified contact time.

10.4.5 After the last carrier of a set (5 total carriers) has been treated with the disinfectant, and the contact time is complete, aseptically transfer carriers into tubes containing the specified neutralizer within 2 minutes. Transfer carriers according to the method specified in the FDA SOP. This set of neutralizer tubes (5 total tubes) will represent the **Neutralizer**-**Primary Subculture** Treatment. Each tube will be inoculated with one mL of each of the five inoculum dilutions as indicated in Table 1.

10.4.6 Following the last carrier transfer into the neutralizer tube, transfer each carrier into a culture tube containing the secondary subculture medium (e.g., FTM). This portion of the assay is not timed, but the transfers should be made as soon as possible. This set of tubes (5 total tubes) will represent the **Secondary Subculture** Treatment. Each tube will be inoculated with one mL of each of the five inoculum dilutions as indicated in Table 1.

10.4.7 Inoculated Controls.

The **Neutralizer-Primary** Inoculated Control contains five tubes of fresh, unexposed neutralizer-primary media.

The **Secondary Subculture** Inoculated Control contains five tubes of secondary subculture media.

The preparation (media preparation number) of each must be the same as used in the treatments. Each tube will be inoculated with one of five inoculum dilutions as indicated in Table 1.

10.4.8 Uninoculated Controls.

Neutralizer-Primary and **Secondary Subculture** uninoculated Controls. One tube each of uninoculated neutralizer and secondary subculture media will be included in the test and incubated with the other tubes.

10.4.9 Inoculating the Tubes. Inoculate each *inoculated* treatment and control tube with 1 mL of the diluted spore suspension as indicated in Table 1. Seed the tubes following the transfer of all carriers.

Table 1. Seeding Treatments and Control Groups with Five Diluted Spore Suspensions*

Neutralizer-Primary Subculture Treatment	Secondary Subculture Treatment (with Carrier)	Neutralizer-Primary Inoculated Control	Secondary Subculture Inoculated Control	Neutralizer-Primary & Secondary Subculture Uninoculated Controls
1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4 1 mL of 10^{-9} → Tube 5	1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4 1 mL of 10^{-9} → Tube 5	1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4 1 mL of 10^{-9} → Tube 5	1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4 1 mL of 10^{-9} → Tube 5	**Not inoculated** 1 Tube of Neutralizer 1 Tube of Secondary Subculture Media

*1 x 10^{-5} through 1 x 10^{-9}; based on an approx. starting suspension of 10^{8} spores/mL

10.4.10 Incubate tubes for 21 days at 37±1°C. For all tubes showing no growth after 21 days, heat shock for 20 minutes at 80±1°C and reincubate for 72±2 hrs.

10.5 Results are recorded as + (growth) or 0 (no growth). Record results on

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Results Form (see 16.0). Confirmation testing of the growth will be performed as follows:

10.6 Identification and Confirmation Testing:

10.6.1

- A minimum of one positive tube per treatment and control, if available, should be confirmed using Gram staining and selective media. If further confirmation is deemed necessary (e.g., presence of contamination in the test system) Vitek analysis may also be used.

- For each treatment and control group, select the tube with growth inoculated with the dilution with fewest CFU/mL delivered and conduct confirmation testing on a sample of the growth.

10.6.2 Gram stains are performed on smears taken from the positive culture tubes. In most cases, *B. subtilis* will be the test microbe. Gram stain for *B. subtilis* is gram positive rod. For the additional confirmatory tests, a loopful of broth from each selected culture tube is streaked on TSA and incubated for 24±2 hr at 37±1°C. Growth on TSA is observed after 24 hours. Colonies of *B. subtilis* are opaque, rough, dull, round, irregular margins, and low convex. Colonial variation may be observed and is typical for this strain.

10.6.3 Record confirmation results on the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Microbe Confirmation Sheet (see 16.0).

10.7 Interpretation of Results.

10.7.1 Plate count data. The plate counts are an essential element of this assay. One of the five dilutions plated should provide counts within the target range, 5-100 CFUs/mL. Neutralizer and subculture media tubes inoculated from this dilution also received this low level of challenge, an aspect critical to the determination of neutralization effectiveness and

bacteriostatic activity.

10.7.1.1 The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of inoculum (spores) is added to the subculture tubes.

10.7.2 Controls. Growth in the **Secondary Subculture** inoculated Control verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. *No growth or only growth in tubes which received high levels of inoculum (e.g., a dilution with plate counts which are too numerous to count) indicates poor media performance.* Growth in the **Neutralizer-Primary** inoculated Control should be comparable to the Secondary Subculture inoculated Control if the neutralizer is the same as the secondary subculture media.

There may be cases when the neutralizer is significantly different from the secondary subculture media. In these cases, growth may not be comparable to the Secondary Subculture inoculated Control.

The **Neutralizer-Primary** and **Secondary Subculture** uninoculated Control tubes are used to determine sterility, and must show no growth for the test to be valid.

10.7.3 Treatments. The occurrence of growth in the **Neutralizer-Primary Subculture** and **Secondary Subculture** treatment tubes is used to assess the effectiveness of the neutralizer. The neutralizer itself or in combination with the recovery (subculture) medium may exhibit bacteriostatic activity against the test microbe. *No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions. For the neutralizer to be deemed effective, growth must occur in the* ***Secondary Subculture***

treatment tubes which received lower levels of inoculum (e.g., 5-100 CFUs/mL).

11.0 DATA ANALYSIS/CALCULATIONS: None

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the forms indicated in section 16.0. Completed forms are archived in notebooks kept in locked file cabinets in D217. Only authorized personnel have access to the locked files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-01 (see ref. 15.3).

13.0 QUALITY CONTROL:

13.1 The OPP Microbiology Laboratory conforms to 40CFR Part 160, Good Laboratory Practices. Appropriate quality control measures are integrated into each SOP.

13.2 For quality control purposes, the required information is documented on the appropriate form(s) (see 16.0).

13.3 Appropriate quality checks will be performed per the Laboratory's SOPs. These include (see ref. 15.4):

SOP QC-01	Quality Assurance of Purified Water
SOP QC-02	Air/Surface Monitoring of Microbiological Laboratories
SOP QC-03	Glass Washing and Detergent Residue Test
SOP QC-06	Use and Maintenance of Biological Safety Cabinets
SOP QC-07	Monitoring of Water Temperature of Recirculating Chillers
SOP QC-08	Monitoring Temperature/Humidity of the Disinfectant Sample Storage Room
SOP QC-09	Establishment of Control Numbers a for Laboratory Supplies
SOP QC-10	Expiration Time and Examination of Media and Reagents
SOP QC-11	Performance Assessment and Sterility Verification of

Prepared Media and Reagents
SOP QC-12 Sterility Check of Pre-Sterilized and Autoclaved Laboratory Supplies
SOP QC-13 Performance Verification of Autoclaves
SOP QC-15 Media and Reagent Preparation: Assigning Prep and Sterilization Run Numbers
SOP QC-17 VITEK: Quality Control Procedures

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Any deviation from the standard protocol and the reason for the deviation will be recorded on the appropriate record sheet (see 16.0); corrective action will be expeditious.

15.0 REFERENCES:

15.1 AOAC Sporicidal Activity Test (Reference: Official Methods of Analysis. 1990. 15th Ed., Association of Official Analytical Chemists, Arlington, VA, Method 966.04). Test methodology is described in detail in the 1993 FDA SOP: FDA Protocol for Testing Sporicidal Activity.

15.2 Cottrill, M. 2000. SOP MB-01-02 Biosafety in the Laboratory.

15.3 LaSota, L. 2000. SOP ADM-01-01 Preparation and Review of Disinfectant Performance Reports, Section 12.0.

15.4 OPP Microbiology Laboratory Standard Operating Procedure (SOP) Manual. 2001. Control Copy Number 1.

16.0 FORMS AND DATA SHEETS:

16.1 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Information Sheet

16.2 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Results Form

16.3 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Time Recording Sheet for Carrier Transfers

16.4 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Microbe Confirmation Sheet

16.5 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Serial Dilution/Plating Tracking Form

16.6 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Inoculum Enumeration Form

Information Sheet for the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test)

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		SOP(s)	
Product Name		Test Date	
Product Sample No.		Neutralizer	
Product Lot No.		Comments:	
Expiration Date			

TEST PARAMETERS/Confirmed by:_____			
Diluent	Specified	Diluent Used	Hardness Date/Init.
			/ /
Organic Soil	Specified	As Prepared/Date/Init.	
Neutralizer	Specified		
Temperature (°C)	Specified	Chiller Unit Display	Test Tube Waterbath
		Before: After:	Before: After:
Contact Time (minutes)	Specified	As Tested	
Carriers (Unseeded)	Control #		Preparation #
	Type:		

TEST MICROBE INFORMATION/Confirmed by:_____	
Test Microbe	
Org. Control No.	

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Results Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Product Name		Neutralizer	
Sample No.		Comments:	

TEST RESULTS*: Date Recorded/Initials:_____

Treatments/Controls	Inoculum Dilutions				
	$1 \times 10^{-}$	$1 \times 10^{-}$	$1 \times 10^{-}$	$1 \times 10^{-}$	$1 \times 10^{-}$
Neutralizer-Primary Subculture Treatment					
Secondary Subculture Media Treatment (with Carrier)					
Neutralizer Inoculated Control					
Subculture Media Inoculated Control					
Neutralizer Uninoculated Control Tube					
Subculture Media Uninoculated Control Tube					

*+ = growth, 0 = no growth

SUMMARY OF RESULTS: Date/Initials:_____

Bacteriostatic Effect Observed?	Yes_____ No_____
Comments:	

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test): Time Recording Sheet for Carrier Transfers

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
EPA Reg. No.	
Product Name	
Sample No(s).	
Organism(s)	
Neutralizer(s)	
Carrier Type	

Initials /date	Disinfectant Tube No.	Carrier No.	Carrier Drop Start Time for carriers (into the disinfectant)		Carrier Drop End Time for carriers (into the neutralizer)		Carrier Transfer (into secondary media)
			Clock	Timer*	Clock	Timer	Start Time[1]
	1						
	2						
	3						
Comments: * = ± 5 seconds, P= Porcelain carriers, SL= Suture loops, BS= *Bacillus subtilis*, FTM = Fluid thioglycollate medium							

1 Carrier transfer into FTM; taken from clock

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Microbe Confirmation Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	
Sample No.		Carrier Type[3]	

Source: Tube/Plate ID	Date/ Initials	Stain Results[1]	Media Information			Results		
			Type	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	API Test / Vitek ID(if applicable)[2]

1 Record Gram Stain results as GPC=gram positive cocci, GNR=gram negative rods, GPR=Gram positive rods.
2 API numerical profile number or Vitek Identification Number 3. Porcelain penicylinders (P) or Suture loops (SL)

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Serial Dilution/Plating Tracking Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Neutralizer(s)	
Sample No.		Organism Control #	

Confirmed by:_____	Dilution Tube								
	1	2	3	4	5	6	7	8	9
Vol. In Dil. Tube prior to Addition									
Volume Added to Dil. Tube									
Overall Dilution in Dil. Tube									
Volume Plated									
Overall Dilution on Plate									
Number of Plates per Dilution									
Media Plated Onto									
Comments:									

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Inoculum Enumeration Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Organism	
Sample No.		Sample No.	

RESULTS: Date/Initials:_____			
Plating Method			
	CFU per Dilution Plate		Average CFU per mL
Dilution	Plate 1	Plate 2	
$1 \times 10^{-}$			
$1 \times 10^{-}$			
$1 \times 10^{-}$			
$1 \times 10^{-}$			
$1 \times 10^{-}$			
TNTC= Too Numerous To Count			
Comments:			

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.